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Actions of Gamma Radiation on Enzymes and Enzyme Systems

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Effects of γ-irradiation on several enzymes, coenzymes and multi-enzyme systems were studied *in vitro*. Crystalline urease, yeast alcohol dehydrogenase (ADH), and liver glutamic dehydrogenase (GDH) and catalase, diphosphopyridine nucleotide (DPN) and succinic acid oxidase system were chosen to be investigated.

γ-Irradiation on urease preparations in their aqueous suspension was proved to cause inactivation of the enzyme by indirect action of radiation products of water.

Among many radiolyzed products of water molecule, hydroxyl and hydroperoxyl radicals and hydrogen peroxide molecules were especially effective to cause inactivation of yeast alcohol dehydrogenase in air-containing aqueous solutions by γ-irradiations.

Conditions which affect the inactivation of enzymes and a coenzyme were studied. The inhibitions of enzyme activities were proved to occur exponentially to the doses of γ-rays. G-value of the inactivation of enzyme depended upon the concentration of enzyme solutions. Sulfhydryl enzymes such as urease, alcohol dehydrogenase were observed to be more radiosensitive to the inactivation than other enzymes in which SH groups are not essential to their activity. Urease was found to be relatively radio-resistant to the inactivation at its optimal pH. In the solutions of DPN from which dissolved oxygen were expelled, characteristic increases of absorbance at 340 mμ were observed in the presence of ethylalcohol or lactate.

Previously added thiol compounds such as cysteine and reduced glutathione were found to be able to protect the inactivation of sulfhydryl enzymes by γ-irradiation. When they were added to the inactivated preparations after irradiation, they could recover the lost activities. By chemical determination of sulfhydryl groups lost, the inactivation of sulfhydryl enzymes by moderate doses of γ-radiation was demonstrated to be oxidation of thiol linkage to disulphide as proposed by Barron.

Radiolytic damages of enzymes and coenzyme by γ-irradiation were studied quantitatively. Changes of absorption spectra of GDH and DPN were found to run with inactivation of the enzymic activities closely. When DPN was irradiated by relatively large doses of γ-rays, splitting of inorganic phosphate and other chemical changes occurred were confirmed by ion exchange chromatography and other chemical methods.

Biochemical effects of γ-irradiation on enzyme systems were studied. A holosystem of apo-glutamic dehydrogenase and co-enzyme DPN was affected by γ-irradiation. The apoenzyme was proved to be more radiosensitive than the coenzyme and additive effects were also observed under the conditions. In the multi-enzyme system, succinic oxidase system in "GREEN'S brei" of fresh mouse liver, succinic acid de-

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hydrogenase system was proved to be more radiosensitive than cytochrome c oxidase system.

INTRODUCTION

Actions of radiation on living cells are attributed to biochemical effects caused by ionizing radiations on certain constituents or their systems of biological importance, especially on enzymes and enzyme systems. It has been well established that there are two modes of actions of radiation, direct and indirect, with regard to the chemical events caused by irradiation.

Dale[1,2] found in his pioneer works about the inactivation of carboxypeptidase and other enzymes by X-irradiation that the enzyme was inhibited in its sufficiently diluted solutions even by relatively small doses of X-rays. He postulated that the inactivation of enzymes was caused by the action of radiation products of water. Therefore, it would be said to be indirect action of the radiation on enzymes in aqueous solutions. Chief radiolyzed products of water dissolving oxygen are hydroxyl radical (OH), hydroperoxyl radical (O_2H) and hydrogen peroxide molecule (H_2O_2)[3]. They are all powerfull oxidizing agents to solutes. As proteins are known to be very sensitive to oxidation, most of enzymes are also oxidized to be inhibited their activities by irradiation. Barron and his collabolators[4,5] demonstrated with many enzymes that enzymes whose active groups are said to be sulfhydryl groups were very susceptible to inhibition by moderate doses of X-rays, owing to oxidation of sulfhydryl groups into inactive disulfide form by the radiolyzed products of water molecules. Besides oxidation of thiol groups many other chemical changes are expected to occur in enzyme molecules such as libration of ammonia [6~8], of carbon dioxide and of hydrogen sulfide and breakdown of peptide linkage, by relatively larger doses of radiations. With regard to irreversible inactivation of enzymes, which are attributed to denaturation of protein by irradiation of larger doses of X-rays, various chemical changes other than oxidation of thiol groups should be expected to occur[9].

In this paper are presented the results of experiments on inactivations of sulfhydryl enzymes[10,11], a non-sulfhydryl enzyme, a coenzyme, and enzyme systems and biochemical approach of this problem is discussed.

EXPERIMENTAL

(1) Materials

Preparation of enzyme. Three sulfhydryl enzymes, urease, yeast alcohol dehydrogenase (ADH), liver glutamic dehydrogenase (GDH), and a non-sulfhydryl enzyme, catalase, were used as specimens in this experiment. Sources and specific activities of the enzymes, and the methods used for the preparation are tabulated in Table 1. The methods of preparation are almost same as described in previous reports[7,12,13] except crystalline urease being in an octahedral form when it was recrystallized by the method reported by

Table 1. Preparation of enzymes and a coenzyme used.

Preparation		Source	Activity or purity	Author and Ref. of preparation method
Sulfhydryl enzyme	Urease	Jack bean meal (Japanese species)	6.8×10^4 Sumner units	Sumner[16] Hellerman[17] Dounce[18] Kobashi[14]
	ADH	Baker's yeast	5.0×10^7 units/g of protein	Racker[19] Iwatsubo[20]
	GDH	Beef liver	1.1×10^6 〃	Olson[21] Strecker[22] Iwatsubo[23]
Non-sulfhydryl enzyme	Catalase	Beef liver	7.3×10^3 Kat. f.	Kitagawa & Shirakawa[24]
Coenzyme	DPN	Baker's yeast	94%	Kornberg[25] Okunuki[26]

Kobashi[14].

Preparation of coenzyme. A well known coenzyme of dehydrogenases, diphosphopyridine nucleotide (DPN), was used as a specimen in this experiment. Source, purity based on its coenzymic activity, and the methods used for the preparation are also tabulated in Table 1.

Isolation of enzyme system. Succinic acid oxidase system which composed of succinic acid dehydrogenase, cytochromes, and cytochrome oxidase in freshly prepared "GREEN's brei" of mouse liver was chosen as a representative of enzyme system. "GREEN's brei" of mouse liver was prepared by Keilin's method[15] with modifications.

(2) Methods

Determination of activities of enzyme, coenzyme and components of enzyme system. The activity of urease was determined according to Sumner's method with modifications using a Beckman's GS pH meter. The procedure was described in the previous paper[8]. The activity of alcohol and glutamic dehydrogenases and also of its coenzyme was measured colorimetrically according to a usual method using a Beckman's DU spectrophotometer as described in the previous paper[12]. The enzyme activity of the single component of the succinic oxidase system was assayed at 30°C in a Warburg apparatus, measuring the speed of O_2-uptake.

Irradiation technique. In this experiments three apparatuses were used for irradiation of γ-rays from ^{60}Co ; one of them was the Toshiba's Teletherapy Unit (Model RIT-1)* equipped with 50 C of ^{60}Co which dose rate is 53.4 kr/hr ±5%, second one was the Toshiba's Irradiation Apparatus** equipped with 200 C of ^{60}Co and the dose rate was 18.9-72.8 kr/hr±1%, and the last one was the 2 KC ^{60}Co irradiation facility*** having about 2000 C ^{60}Co with the dose rate of

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234 kr/hr±5%. Radiation dose was determined by a chemical dosimetry using ferrous sulphate[27]. At calculation of the dose rate, an ionic yield of 15.5 mole ferric ion per 100 eV was used. The irradiation was carried out at 0°C or at room temperature under atomospheric air (25~27°C) in the dry state, in the aqueous solutions, or in adequate buffer solutions.

Determination of sulfhydryl groups. The amount of sulfhydryl groups in enzyme proteins were determined by Boyer's colorimetrical method[28]. A new method devised by Ozawa and Egashira was also applied for sulfhydryl determination[29,13].

Measurements of the activity of enzyme systems. The activity of enzyme systems was determined by measuring the rate of oxygen uptake by the Warburg's manometric apparatus as follows: Complete succinic acid oxidase system was composed of 0.5 ml of 0.5 *M* sodium succinate, 0.2 ml of 1 per cent reduced cytochrome c solution, 1.0 ml of 0.3 *M* phosphate buffer pH 7.0, 0.8 ml of water and 0.5 ml of the enzyme preparation. Complete succinic dehydrogenase system was formed from 0.5 ml of 0.5 *M* sodium succinate, 1.3 ml of 0.1 per cent methylene blue solution, 0.4 ml of 0.1 *M* potassium cyanide, 0.8 ml of 0.3 *M* phosphate buffer pH 7.0 and 1.0 ml of the enzyme preparation. Complete cytochrome c oxidase sestem was composed of 0.5 ml of 1.0 per cent reduced cytochrome c, 1.0 ml of 0.3 *M* phosphate buffer pH 7.0, 1.0 ml of water and 0.5 ml of the enzyme preparation.

RESULTS

(1) Direct and Indirect Actions of γ-Irradiation on Urease

Effects of γ-irradiation on ureolytic activities of several preparations of urease were examined and the results obtained are given in Table 2. It was found that a purified enzyme preparation was more sensitive to inhibition of the activity by γ-irradiation than a crude preparation and intact specimens. Crystalline urease in its aqueous solution was completely inactivated, while in

Table 2. Direct and indirect actions of γ-irradiation on various urease preparations.

Preparation	Amount mg (mg/0.5ml)	Activity Sumner unit	Inactivation, % Dose, kr.				
			2.5	5.0	17	50	800
Jack bean meal	50	170	—	—	0	—	18
〃 suspended in water	(50)	200	—	—	0	—	50
Crude urease solution	(10)	430	7	25	32	54	100
Purified 〃	(5)	490	23	45	100	100	100
Crystalline urease	3.6	356	—	7	—	—	—
Crystalline urease solution	(3.4)	312	—	100	—	—	—

Dose rate : 520 r./min., irradiated at room temp. 20~25°C.

the dried state the same preparation was proved to be much resistant to the inactivation by the same dose of γ-rays.

(2) Inhibitory Entities in Aqueous Solutions of Enzymes

Three inhibitory entities, which would be produced in the aqueous solution containing oxygen by γ-irradiation, were proved to inactivate yeast alcohol dehydrogenase. The results obtained are shown in Table 3. A ratio of three

Table 3. Inhibitory effects of HO, HO_2 and H_2O_2 to yeast alcohol dehydrogenase.

Reaction system	Activity, unit		Inhibition %	Effective entity
	Before irradiation	After irradiation		
ADH[1]+γ-Rays	103	45	56.0	HO, HO_2, H_2O_2
ADH[3]+γ-Rays	140	81	42.8	〃
ADH[1]$-O_2(+N_2)$	103	89	13.6	(N_2)
ADH[1]$-O_2(+N_2)+\gamma$-Rays	103	62	39.8	HO
ADH[2]+$FeSO_4$	115	113	1.7	($FeSO_4$)
ADH[2]+H_2O_2	115	105	8.7	(H_2O_2)
ADH[2]+$FeSO_4+H_2O_2$	115	0	100	HO
ADH[3]+Boiled catalase $+\gamma$-Rays	140	85	39.3	HO, HO_2, H_2O_2
ADH[3]+Catalase+γ-Rays	140	101	27.8	HO, HO_2
ADH[1]+H_2O_2+Catalase	102	100	1.9	—

1, 2, 3, indicate the concentration of ADH solution. 1, 0.10 mg/ml ; 2, 0.12 mg/ml ; 3, 0.14 mg/ml ; As dose of γ-rays 30 kr was used ; Concentration of $FeSO_4$ solution is 0.25 μM and that of H_2O_2 is 25 μM ; N_2 gas was passed through the solution for 20 min.

entities in their effect to the enzyme was calculated from the results and was found to be 47 : 29 : 24 to OH : O_2H : H_2O_2 under the examined conditions.

(3) Relation between Irradiating Conditions and Inactivation of Enzymes or Coenzyme

(i) The dose effect. Yeast alcohol dehydrogenase, urease, liver glutamic dehydrogenase and catalase as well as diphosphopyridine nucleotide, were irradiated with γ-rays in their aqueous solutions which contained dissolved oxygen. Inhibitions of their enzyme activities were observed to vary with the doses of γ-rays. The results are presented in Table 4. The inhibition was proved to occur exponentially to the doses as shown in Fig. 1.

It was found that sulfhydryl enzymes, yeast alcohol dehydrogenase, urease, and liver glutamic dehydrogenase, were inactivated easily in their aqueous solutions by relatively moderate doses of γ-rays, while the activity of non-sulfhydryl enzyme, catalase, was found not so easily to be inhibited by 5,000 r of γ-ray dose under the experimental conditions.

Reaction yield of the inactivation of enzymes by γ-irradiation was 1.42 for crystalline urease, 0.01 for liver glutamic dehydrogenase and 0.006 for diphos-

Table 4. Inactivation of enzymes and a coenzyme in aqueous solutions by γ-irradiation.

		Dose, r	Inactivation, %	Concentration
Sulfhydryl Enzymes	Yeast alcohol dehydrogenase	400	7.4	1 μg/0.2 ml., 54 units
		800	33.2	
		6,000	53.7	
		10,000	74.2	
	Liver glutamic dehydrogenase	1,800	6.6	90 μg/0.1 ml., 108 units
		8,000	13.2	
		12,500	46.2	
		200,000	76.4	
	Urease	800	0	
		2,500	22.9	5 mg/0.5 ml
		8,300	45.8	480 Sumner units
		17,000	100	
Non-sulfhydryl Enzyme	Catalase	5,000	0	5 mg/ml
Coenzyme	Diphospho-pyridine nucleotide	30,000	0	100 μg/ml, 118 units
		60,000	10.5	
		120,000	29.8	
		260,000	50.8	
		480,000	80.7	

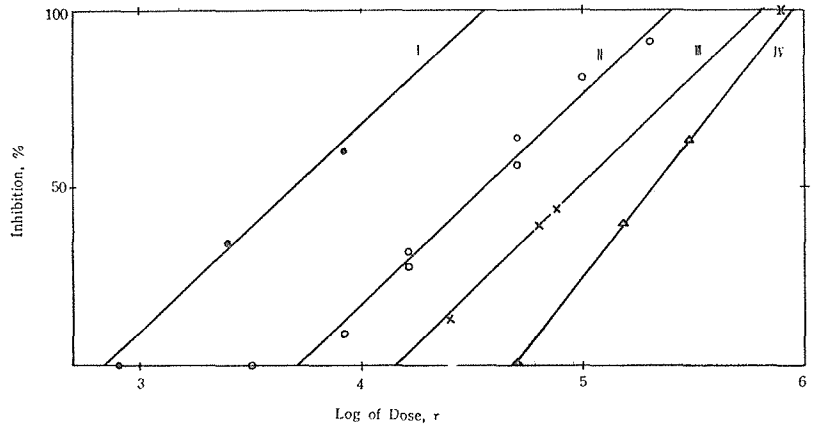

Fig. 1. Inactivation of enzymes and a coenzyme in aqueous solutions by γ-irradiation. I, Crystalline urease 4 mg/0.5 ml ; II, Crude urease preparation 5 mg/0.5 ml ; III, Glutamic dehydrogenase preparation 900 μg/ml ; IV, Diphosphopyridine nucleotide preparation, 50 μg/ml ; Dose rate, 5×10^4 r/hr.

phopyridine nucleotide respectively.

(ii) The concentration effects. Inactivation of enzymes in various concentrations of enzyme solutions by γ-irradiation was examined and characteristic results were observed as shown in Fig. 2.

In urease the inactivation was observed to depend linearly upon the concentration of the solutions, while in liver glutamic dehydrogenase the inhibition

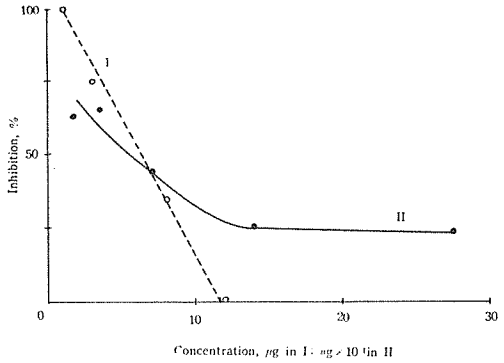

Fig. 2. Inactivation of urease (I) and GDH (II) in various concentration of solutions by γ-irradiation.
Dose, 8.3kr in I ; 5.3 kr in II.

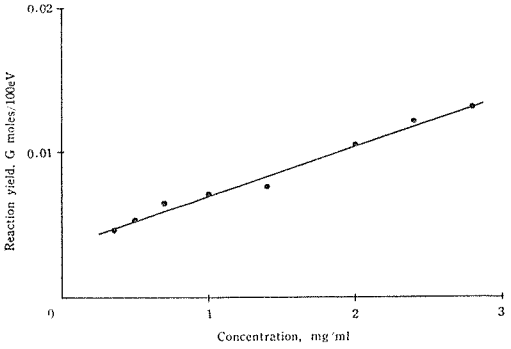

Fig. 3. Reaction yield of inactivation of glutamic dehydrogenase in various concentration of solutions.
Dose ; 5×10^4 r.

of its activity was variable to the concentration of the solutions.

The reaction yield of the inactivation of liver glutamic dehydrogenase was seen to vary with the concentration of its solutions as shown in Fig. 3., while G-values of DPN calculated from the charges of its absorbance at 260 mμ were almost constant.

(iii) The pH effect. Inhibition of ureolytic activities in various pH of urease solutions was observed to show minimum value at optimum pH of the enzyme. The determined activities are shown in Fig. 4.

(iv) The protective effect of chemicals. The inactivation of enzymes by

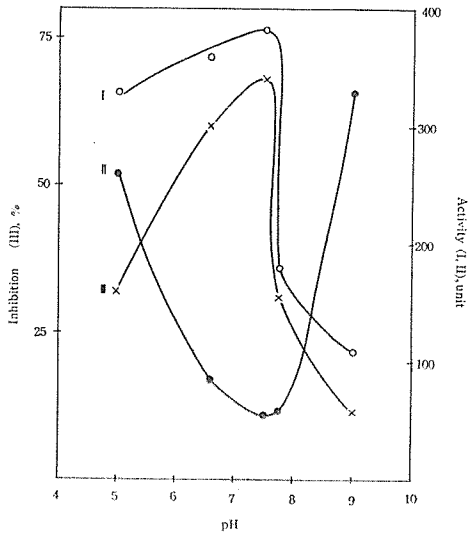

Fig. 4. Inactivation of urease in various pH solutions by γ-irradiation. Crystalline urease 4 mg/0.1 ml ; 0.1 *M* phosphate buffer solution except 0.1 *N* acetate buffer at pH 5.00 and 0.2 *M* borate buffer at pH 9.08, 0.4 ml; Dose, 8 kr; I : Activity before irradiation, II : Activity after irradiation, III : Inhibition.

Table 5. Protecting effect of various compounds to enzyme inactivation by γ-irradiation.

Enzyme	Protecting compound*	Conc. *M*	Protection %	Protecting Power, *Q***
Urease[1]	Reduced glutathione	0.004	87	6.34
	Egg albumin	0.011	33	0.106
	Glycine	0.6	25	0.075
	Sodium chloride	0.85	8	0.019
Liver Glutamic Dehydrogenase[2]	Reduced glutathione	0.004	—	1.3
	DPN	0.016	—	0.4
	Phenylalanine	0.5	—	0.02
	KH_2PO_4	0.2	—	0.01
	Thiourea	0.5	—	0.006
	Na_2SO_4	0.5	—	0.005
	Ethylalcohol	3.0	—	0.003

* In 0.4 ml. ; Dose 53.4 kr (/kr)

** Protecting power Q was difined by Dale[30] as :

$$Q = \frac{{}_{37}D_{p+e} - {}_{37}D_e}{{}_{37}D_e} \times \frac{W_e}{W_p},$$

where ${}_{37}D_{p+e}$ is the dose required to cause 37 per cent inhibition of initial enzyme activity when the compound is added to the enzyme solution to be irradiated, ${}_{37}D_e$ is the same dose without the compound, W_e is weight of enzyme, 90 μg containing the activity, 1,200 units per protein mg in 0.1 ml of the solution, and W_p is a amount of the compound added to be irradiated.

1. Crystalline urease 4 mg/0.1 ml, 357 units
2. 75 μg/0.1 ml, 1,200 units/mg

γ-irradiation was found to decrease by addition of various compounds into the enzyme solutions. The effects observed were shown in Table 5. Reduced glutathione which contains thiol group in its molecule, was most effective to

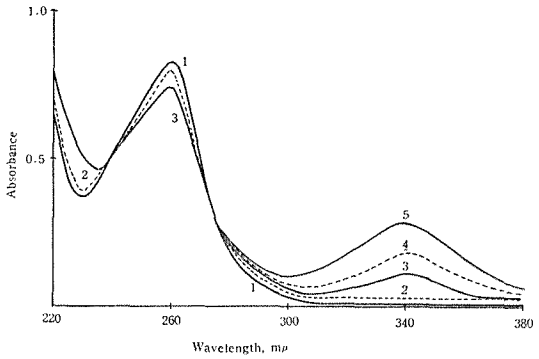

Fig. 5. Ultraviolet absorption spectra of γ-irradiated DPN in air-free aqueous solution, and in the presence of catalytic compounds.
DPN, 90 μg/ml ; Dose 5×10^4 r ; 0.1 M pyrophosphate buffer pH 8.7
1. Non-irradiated DPN
2. Non-irradiated DPN containing dissolved hydrogen
3. γ-irradiated DPN in the presence of 0.01*M* Li-lactate
4. γ-irradiated DPN in the presence of 0.5 *M* ethyl alcohol
5. Enzymatically produced DPNH by ADH after γ-irradiation in 0.5 *M* ethyl alcohol, 4.

this protecting action of the enzyme inactivation.

(v) **The effect of dissolved oxygen to coenzyme solutions.** When DPN was irradiated by γ-rays with a catalytic amount of ethylalcohol in its aqueous solutions from which dissolved air had been excluded by apropriate bubling of hydrogen gas under diminished pressure, light absorption was observed to appear at 340 mμ similar to that of reduced DPN as shown in Fig. 5.

Besides ethyl alcohol, lactate was found to be also effective but glycine, glutamic acid and hydrogen were not effective. Reaction yield is given in Table 6. Substance produced, however, was proved to be unable to reduce acetaldehyde with apoenzyme of alcohol dehydrogenase and also does not emit any fluorescence contrary to enzymatically produced DPNH.

Table 6. Effects of γ-irradiation on DPN in oxygen-free aqueous solutions.

Added Compound	Absorbance, $E_{340m\mu}$	G ($E_{340m\mu}$)*
0.5 *M* Ethanol	0.105	0.34
0.01 *M* Lithium lactate	0.090	0.28
0.01 *M* Sodium glutamate	0.020	0.06
〃 Glycine	0.025	0.08
〃 Cystine	0.000	0.00
Hydrogen	0.025	0.08

* DPN moles/100 eV.

(4) Inhibitory Effects on Biologically Active Thiol Compounds and Sulfhydryl Enzymes by γ-Irradiation

It has been shown in Table 5 that thiol compound, such as reduced glutathione, was effective to protect the inactivation of enzymes irradiated by γ-ray. Thiol compounds themselves were known to be remarkably susceptible to radiolytic damages by γ-irradiation and the thiol group of cysteine was proved

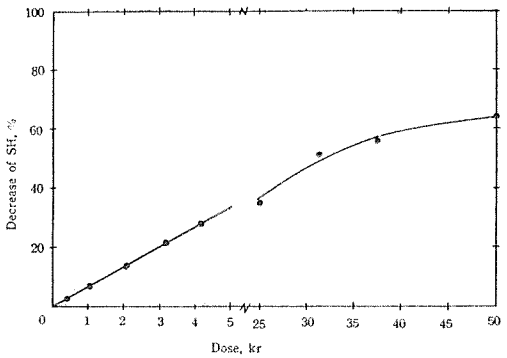

Fig. 6. Changes of the amounts of SH groups of cysteine by γ-irradiation. Cysteine 1×10^{-3} *M* at pH 7.0 (below 5 kr) and pH 8.0 (over 25 kr)

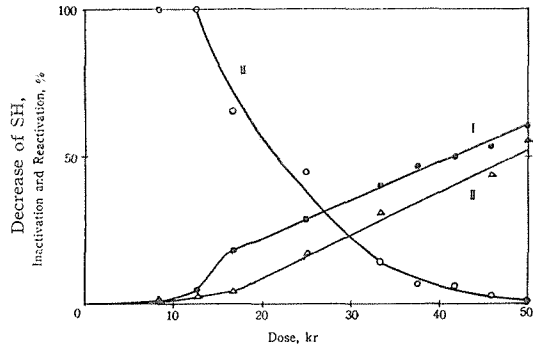

Fig. 7. Inactivation (I) of urease and decrease of the amounts of SH groups (II) in its molecules by γ-irradiation and reactivation (III) by the addition of cysteine.
Crystalline urease 1.0 mg/0.5 ml, 100.0 units; Cysteine 1×10^{-2} *M*.

to be easily oxidized by γ-irradiation in its aqueous solution as shown in Fig. 6. Similar disappearance of sulfhydryl groups and corresponding inactivation were observed in the aqueous solutions of sulfhydryl enzymes such as urease, yeast alcohol dehydrogenase and glutamic dehydrogenase. The experimental results obtained are shown in Table 7 and Fig. 7.

When thiol compounds such as cysteine and reduced glutathione were added to the enzyme solutions in which the enzymes had been more or less inactivated after γ-irradiation, the inhibited activities were partly recovered. The experimental results on urease and GDH are shown in Fig. 7 and Table 8. The reactivation of inactivated enzymes was observed only when the relatively moderate doses of γ-rays was irradiated.

Table 7. Decrease in the amounts of sulfhydryl groups of enzyme proteins by γ-irradiation.

Enzyme	Dose, r	Amount of SH groups, *M*	Decrease %	Inactivation %	Method of detn.
Y. ADH[1]	0	5.38×10^{-8}	—	—	Boyer's[28]
	3.0×10^{4}	4.81×10^{-8}	10.6	—	
Y. ADH[1]	0	1.71×10^{-5}	—	—	Ozawa's[29]
	3.0×10^{4}	1.01×10^{-5}	41.3	—	
L. GDH	0	1.52×10^{-7}	—	—	
	5.0×10^{2}	1.34×10^{-7}	11.7	—	
	6.0×10^{3}	1.29×10^{-7}	14.9	—	Boyer's[28]
	1.5×10^{4}	1.23×10^{-7}	19.1	—	
Urease[2]	0	2.32×10^{-5}	0	0	
	5.0×10^{2}	1.96×10^{-5}	15.5	16	
	5.0×10^{3}	1.42×10^{-5}	38.9	32	Ozawa's[29]
	1.5×10^{4}	1.26×10^{-5}	45.8	36	
	3.0×10^{4}	1.13×10^{-5}	51.3	48	

1, 14 μg/0.1 ml ; 2, 0.6 mg/0.1 ml

Table 8. Reactivating effects of thiol compounds to the inactivated enzymes by γ-irradiation.

Reactivating compounds	Enzyme	Dose, kr	Inactivation %	Reactivation %
CSH[1]	Urease[2]	0.8	0	100
		2.5	23	55
		8.3	46	14
GSH[3]	Urease[4]	16.6	16	100
		25.0	36	100
		50.0	57	0
GSH[3]	GDH[5]	1.8	7	100
		12.5	46	19

1, Cysteine 0.01 *M*; 2, Purified preparation 5 mg/0.5 ml, 480 Sumner units; 3, Reduced glutathione 0.002 *M*; 4, Crude preparation 5.5 mg/0.5 ml, 280 Sumner units; 5, Crude preparation 75 μg/0.1 ml, 91 units.

(5) Radiolytic Effects on Enzymes and a Coenzyme by γ-Irradiation

Ultraviolet absorption spectra of glutamic dehydrogenase and diphosphopyridine nucleotide were observed to change in their characteristic absorption at 260 mμ and other wave lengtn as shown in Fig. 8 and 9. It was found that there are parallel relationship between the decrease of the absorbance and the inactivation of enzymic activities. The results obtained are presented in Table 9.

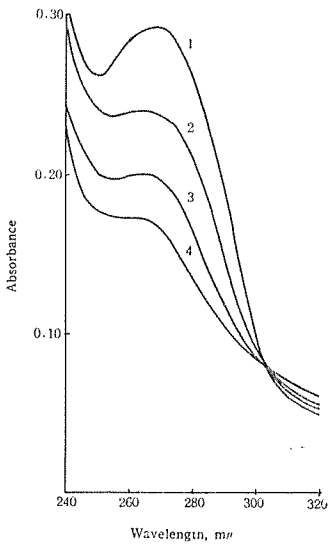

Fig. 8. Changes of ultraviolet absorption spectra of GDH by γ-irradiation.
1. Non-irradiated
2. 100 kr irradiated
3. 200 kr 〃
4. 425 kr 〃
GDH 275 μg/ml ; Dose rate 5×10^4 r/hr at 0°C.

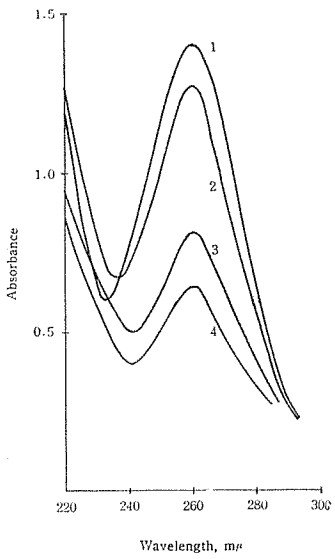

Fig. 9. Changes of ultraviolet absorption spectra of DPN by γ-irradiation in various concentrations of aqueous solutions.
1. Non-irradiated
2. 396 μg DPN/ml
3. 79 〃
4. 39 〃
Dose : 8×10^5 r

Table 9. Changes of ultraviolet absorption spectra and activities of DPN by γ-irradiation.

Dose, kr	Inactivation %	Change of Absorbance ΔE, % −ΔE max (260 mμ)		+ΔE min (232 mμ)		Diff. ΔE, % ΔE max −ΔE min		Ratio ΔE, % ΔE max/ ΔE min	
50	0	1.5		14.4	—	12.2	—	14.0	—
100	10.5	12.4		15.7	—	31.2	—	24.1	—
200	29.8	11.7		34.6	32.2*	42.6	40.9*	34.3	32.9*
425	50.8	15.4		42.8	37.2**	54.4	50.6**	40.4	38.1**
800	80.7	31.8		36.5	22.6**	77.6	68.4**	50.2	44.2**

* Min. 235 mμ, Diff. ΔE 260 mμ −ΔE 235 mμ, Ratio ΔE 260 mμ/ΔE 235 mμ.
** Min. 238 mμ, Diff. ΔE 260 mμ −ΔE 238 mμ, Ratio ΔE 260 mμ/ΔE 238 mμ.
DPN, 50 γg/ml.

Appreciable amounts of ammonia were found to be librated from the enzyme solutions irradiated by various doses of γ-rays. The amounts of ammonia librated from urease solutions were determined by Conway's microdiffusion method[31] and the results are shown in Fig. 10. Libration of ammonia should be one of the result of radiolytic breakdown of proteins. This breakdown was observed to occur only when larger doses of γ-rays were irradiated, while the reversible inactivation of sulfhydryl enzymes occurred only by relatively moderate doses of γ-rays.

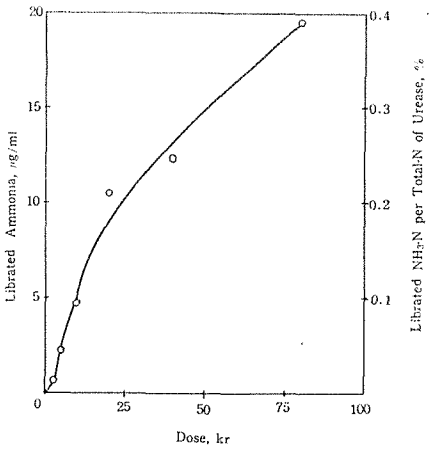

Fig. 10. Ammonia librated from urease solutions by γ-irradiation. Enzyme solution was containing 26.2 mg of crystalline urease per ml.

Splitting of inorganic phosphate was also observed from diphosphopyridine nucleotide solution which was irradiated by γ-rays. The amount of phosphate determined was shown in Table 10. A cyanide test at 325 mμ by which nicotinic acid N-riboside linkage is indicated[32], was examined and the results are presented also in Table 10.

Table 10. Radiolytic damages of DPN by γ-irradiation.

DPN Concentration μg/ml	Inactivation %	Decrease of $E_{325\,m\mu}$ by cyanide test, %	Libration of inorganic phosphate, %
300	52	45	5
60	100	78	14

Radiolytic products formed from the solution of diphosphopyridine nucleotide by γ-irradiation were separated by a ion-exchange chromatography using Dowex-1×8.

The result of radiolysis of DPN, when 7.5 mg of DPN dissolved in aqueous solution was irradiated with 38×10^5 r of γ-dose, was shown as follows: Inactivation of coenzyme activity was 13%, breakdown of nicotinic N-riboside presented by the cyanide test 16%, librated sugar determined by a orcinol reaction 43%, librated phosphate 13%, and decrease of absorbance at 260 mμ 73%.

The radiolysate was separated by chromatography on a Dowex-1 ×8 column (0.9cm×20cm) which has prepared as formate form. Substances absorbed were eluted gradiently from formic acid of 0 to 4 N and effluent fraction I was obtained. The next elution was carried out by using 4 N formic acid and 0.8 M ammonium formate solutions and effluent fraction II was obtained. About 49% of total radiolysate calculated from absorbance at 260 mμ was not absorbed on the column and about 42% was absorbed. This non-absorbed substances did not contain any librated phosphate, while absorbed fraction was found to contain

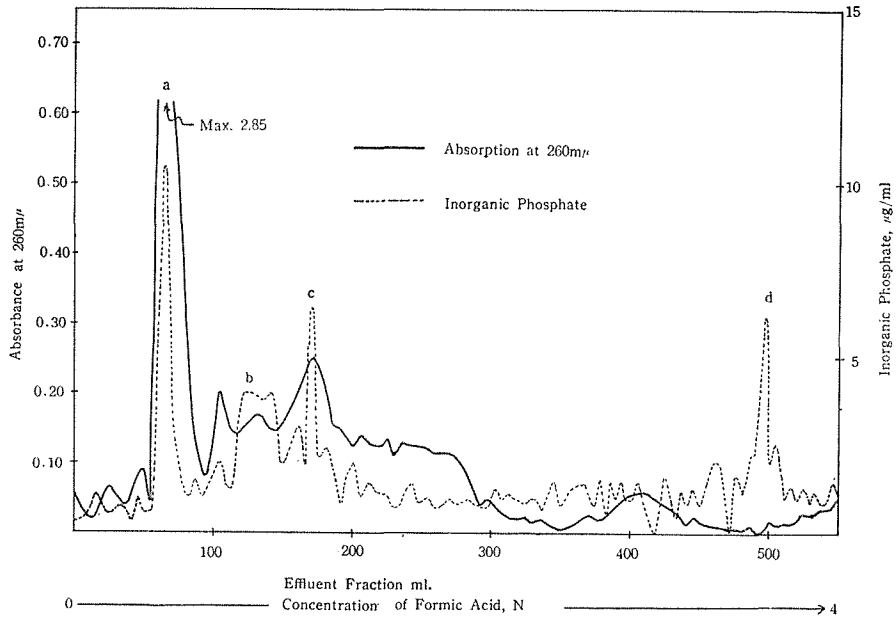

Fig. 11. A ion-exchange column chromatogram of the radiolysate of DPN by γ-irradiation.
DPN 7.5 mg/5 ml, Dose: 38×10^5 r, Dowex 1×8 formate form

all of liberated phosphate; effluent fraction I contained about 43% and effluent fraction II about 49% of total librated phosphate.

Four peaks were observed to appear on a chromatogram as shown in Fig. 11. Peak a was proved to be unchanged DPN by its coenzyme activity with ADH. Peak b and c were shown negative cyanide test, and have not yet characterized successfully. Peak d was determined to be inorganic phosphate, since any appreciable increase in the amount of inorganic phosphate could not found when the elute was decomposed by a perchloric acid reagent.

(6) Biochemical Effects of γ-Irradiation on Enzyme Systems

Effects of γ-irradiation on holo-enzyme composed of apoenzyme of glutamic dehydrogenase and its coenzyme, diphosphopyridine nucleotide, were examined.

Table 11. Effects of γ-rays on holoenzyme systems of glutamic dehydrogenase.

Holoenzyme system	Amount of enzyme μg	Dose $r \times 10^{-4}$	Inactivation of constituent[4] %	Inhibition of the activity of holoenzyme system, %
GDH[1]	125	0.8	0	40
DPN[3]	(50)	15.0	40	
GDH[1]	125	2.5	32	84
DPN[3]	(50)	30.0	60	
GDH[2]	50	6.3	39	39
DPN[3]	(600)	6.3	17	

1. Apoenzyme solution containing 1.25 mg of GDH of 200 units per protein mg per ml was used.
2. Apoenzyme solution containing 0.4 mg of GDH of 1,200 units per protein mg per ml was used.
3. Coenzyme solution containing 1.2 mg of DPN per ml in 0.2 *M* phosphate buffer was used.
4. Holoenzyme solution containing apoenzyme or coenzyme which were not yet irradiated, were used to determine the activity of its constituent of holoenzyme.

Table 12. Effects of γ-irradiation on succinic acid oxidase system of the mouse liver.

Enzyme system	Inhibition of O_2-uptake, %	
	4.2×10^5 r. dose	9.45×10^5 r. dose
Succinic oxidase	16.2	—
	18.2	64.2
Succinic acid dehydrogenase	15.1	—
	13.3	16.5
Cytochrome c oxidase	6.9	—
	5.7	6.7

When a relatively much amount of the apoenzyme to DPN was contained the effect was appeared to be similar to that on the apoenzyme itself. In the case of the amount of coenzyme being superior to the amount of apoenzyme, diphosphopyridine nucleotide was observed to show high resistance to γ-irradiation and the total effects came out as addition of the both effects on apoenzyme and DPN. The results are shown in Table 11.

Irradiation of γ-rays on succinic acid oxidase system prepared from the fresh mouse liver was carried out. γ-Irradiation was observed to cause the influence on oxygen uptake of succinic acid dehydrogenase, cytochrome c oxidase and whole succinic acid oxidase system. From the results presented in Table 12, it was found that among them succinic acid dehydrogenase system was the most sensitive to inhibitory action by γ-irradiation on oxygen uptake.

DISCUSSION

The view of indirect action of ionizing radiations on the sensitive constituent of cells in their aqueous solution may be not inappropriate to explain the phenomena occurred in cells by irradiation. Even in the case of Jack bean meal a few per cent of water is contained and, therefore, the inhibitory effects occurred are not attributed to the direct action of irradiation. When the meal was irradiated in the state of suspension in water, remarkable inactivation of urease was observed to occur. This fact demonstrates that the inactivation is caused by the indirect action of the radiation products of water. The crystalline urease in a aqueous solution, indeed, were also inhibited easily, while the same crystalline enzyme was proved to be very resistant in its dried state by a small dose of γ-rays. By the elimination of the effects caused by the radiolysate of water, only seven per cent of the total inactivation (Table 2) are due to the direct action of radiation. Holmes[33] reported that ribonuclease was affected by these radiation products of water. Barron[34] was obtained similar results on thiol compounds. Evidence for the indirect action of radiation to the aqueous solutions is also given by the exponential relation between degree of inactivation and dose of γ-rays for several enzyme and coenzyme solutions. This relationship was well established in the works of Dale[30] and of Barron[35]. Reaction yields of the enzyme inactivation by γ-irradiation, however, are not so uniform by the conditions of the solution. The G-values of the inactivation of liver glutamic dehydrogenase were variable with the concentration of their aqueous solutions. This fact shows that existence of mixed action of direct and indirect natures would be expected. The similar results have been also obtained in the case of other enzymes[36].

Hydrogen ion concentration of the enzyme solution, which is one of the most important factors for its activity, has been reported to vary the effects of the inhibition of enzymes by radiation. The experiment carried out on urease showed that when the urease solutions were irradiated by γ-rays at its optimum pH, the least inactivation was observed. Since the optimum pH for enzyme activities is variable to the kinds of their buffered solution used,

the effect of the kinds of buffer salts could not be found. Protecting effects of the buffer solution should be considered independently.

The idea of competitive actions of protecting compounds to enzyme molecules was proposed by Dale[30]. Other chemical process for protecting action has not yet elucidated except the protection by oxidation of thiol compounds.

Some enzymes which requires the presence of sulfhydryl groups for activity, have been known to be very susceptible for the inactivation by moderate amounts of X-rays. This inactivation was due to oxidation of the sulfhydryl groups to inactive disulfide form as shown by enzyme reactivation with glutathione. This was confirmed in the case of γ-rays as shown in Fig. 7 and Table 7, in which the results on three sulfhydryl enzymes are presented. Proposed entities which are responsible to the oxidation of sulfhydryl groups should be radiation products of water produced in aqueous solutions of the enzymes by γ-irradiation as mentioned above. Barron[4,5] proposed that the inactivation of sulfhydryl enzymes by three radiation products of X-rays in aqueous solutions should be take place by the following process.

$$2\,ESH + 2\,OH \longrightarrow ES\text{-}SE + 2\,H_2O$$
$$2\,ESH + 2\,O_2H \longrightarrow ES\text{-}SE + 2\,H_2O_2$$
$$2\,ESH + H_2O_2 \longrightarrow ES\text{-}SE + 2\,H_2O$$

Where ESH and ES-SE show reduced (active) and oxidized (inactive) enzyme respectively. In progress of these reactions measurable changes in sulfhydryl groups of the enzyme molecules should be expected to occur in relation to change of activities of the enzyme. Decreases occurred in the amounts of sulfhydryl groups in crystalline enzymes during γ-irradiation, were determined by colorimetric determinations. It was demonstrated that decrease in the amounts of sulfhydryl groups in ADH, GDH and urease preparations came up to the degree of inactivation of these enzymes.

Barron[37] and Swallow[38] reported that when DPN was irradiated with X-rays in oxygen-free aqueous solutions a reduced DPN-like compound having its absorption at 340 mμ was produced. Swallow found that the newly produced compound was differ from enzymatically reduced DPN (DPNH) which shows also fluorescence contrary to Barron's suggestion. The newly produced compound by γ-irradiation is shown in Fig. 5 and was proved to show similar behaviors to Swallow's compound. The product is appeared to be a polymerized form of DPN, which was proposed as a dimer by Swallow[39].

Besides the recoverable inactivation of sulfhydryl enzymes caused by moderate doses of γ-rays, an irreversible inhibition of the activity of enzymes was taken place by larger doses of the radiation as shown in Fig. 7. This type of inactivation is presumed to be due to nonspecific denaturation of protein molecules. In the course of denaturation, enzymes and coenzymes are attacked by direct or indirect actions of radiation. Changes in ultraviolet spectra of a enzyme and a coenzyme showed that their chemical structure was changed by γ-irradiation.

Quantitative determinations of librated fragments from γ-irradiated enzyme and coenzyme were carried out successfully by a column chromatography.

The inactivation of enzyme and coenzyme observed to be followed to libration of ammonia and inorganic phosphate. With regard to these phenomena radiolysis of amino acids, peptides have been investigated and the result will be reported in near future.

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